Chapter-11 Biotechnology:Principles and Processes

Very Short Answers Questions:

1. Define biotechnology?

A: The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services'

2. What are molecular scissors? Where are they obtained from?

A: Restriction enzymes which cut DNA at specific locations are referred as molecular scissors. They are obtained from bacteria.

3. Name any two artificially restructured plasmids?

A: pBR322, pUC 19, 101

4. What is EcoRI? How does it function?

A: EcoRI is a restriction endonuclease obtained from *Escherechia coli* RY_{13} bacteria. It recognizes palindromic sequences 5'GAATTC3' and cuts between G and A

5. What are cloning vectors? Give an example?

A: Vectors used for multiplying the foreign DNA sequences are called cloning vectors.

E.g. Plasmids, Bacteriophages

6. What is recombinant DNA?

A: DNA ligated with fragments of foreign DNA is called as recombinant DNA

7. What is palindromic sequence?

A: Palindromes are groups of letters that form the same words when read in both forward and backward directions. **E.g.** MALAYALAM.

In DNA, palindromes are inverted repeats of base pairs.

E.g. 5'-GAATTC-3'

3'-CTTAAG-5'

8. What is the full form of PCR? How is it useful in biotechnology?

A: Polymerase Chain Reaction.

It helps in amplification of gene and synthesizes multiple copies of DNA of interest.

9. What is down –stream processing?

A: The different steps involved sequentially in a process of industrial synthesis are collectively referred as down steam processing.

10. How does one visualize DNA on an agar gel?

A: Staining the DNA with ethidium bromide followed by exposure to UV radiation shows bright orange coloured bands of DNA.

11.How can you differentiate between exonucleases and endonucleases?

A: Exonucleases remove nucleotides from the ends of the DNA.

Endonucleases make cuts at specific locations within DNA.

Short Answer Questions

1. Write short notes on restriction Enzymes?

Ans: Restriction enzymes belong to a larger class of enzymes called **nucleases**.

These are of two kinds; **exonucleases** and **endonucleases**.

Exonucleases remove nucleotides from the ends of the DNA whereas, endonucleases make cuts at specific positions within the DNA.

Each restriction endonuclease functions by 'inspecting' the length of a DNA sequence. Once it finds its specific recognition sequence, it will bind to the DNA and cut each of the two strands of the double helix at specific points in their sugar-phosphate backbones.

Each restriction endonuclease recognises a specific **palindromic nucleotide sequences** in the DNA.

The palindrome in DNA is a sequence of base pairs that reads same on the two strands when orientation of reading is kept the same. For example, the following sequences reads the same on the two strands in 5′--- 3' direction.

This is also true if read in the 3′ --- 5′ direction.

↓

5′ —— GAATTC —— 3′

3′ —— CTTAAG —— 5′

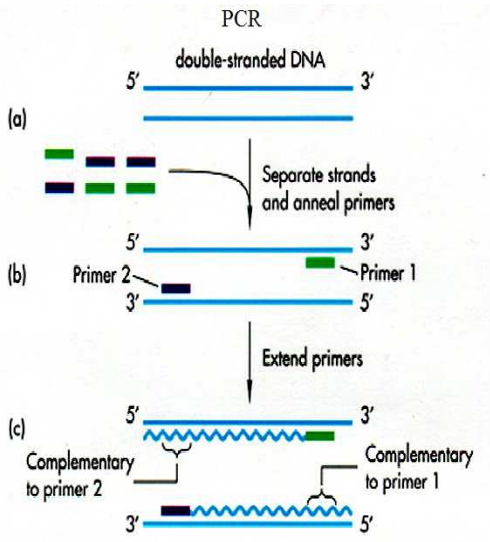
↑

Restriction enzymes cut the strand of DNA a little away from the centre of the palindrome sites, but between the same two bases on the opposite strands. This leaves single stranded portions at the ends. There are overhanging stretches called sticky ends on each strand .

The convention for naming these enzymes is the first letter of the name comes from the genes and the second two letters come from the species of the prokaryotic cell from which they were isolated, E.g., EcoRI comes from *Escherichia coli* RY_{13}. In EcoRI, the letter 'R' is derived from the name of strain. Roman numbers following the names indicate the order in which the enzymes were isolated from that strain of bacteria.

2. Give an account of amplification of gene of interest using PCR?

Ans: PCR stands for **Polymerase Chain Reaction**. In this reaction, multiple copies of the gene or DNA of interest is synthesised *in vitro* using two sets of **primers** and the enzyme **DNA polymerase**. Primers are small stretches of artificially synthesised nucleotide sequences that are complementary to the starting regions of DNA at both ends. **Primers provide 3'OH** group for the extension of the chain of nucleotides in 5'→ 3' direction on opposite complimentary strands.



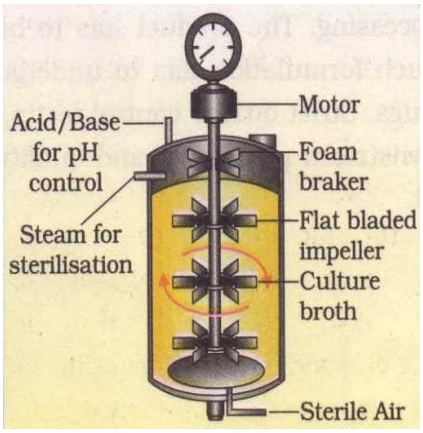
The enzyme extends the primers using the nucleotides provided in the reaction and both the strands of the genomic DNA acts as templates. At high temperatures of around 80^0C both the strands of the DNA separate and acts as templates. At this temperatures amplification is achieved by the use of a thermostable DNA polymerase isolated from a bacterium, ***Thermus aquaticus***, which remain active during the high induced temperature. If the process of replication of DNA is repeated many times, the segment of DNA can be amplified to approximately **billion times**, i.e., 1 billion copies are made. The amplified fragment if desired can now be used to ligate with a vector for further cloning or is used in identification of the DNA.

3. What is a bio-reactor? Describe briefly the stirring type of bio-reactor?

Ans: A bio-reactor is a vessel used as a fermentor to multiply the cells or organisms harbouring the recombinant DNA so that the DNA expresses and produce large amounts of the product in the form of protein, enzyme or any other substance.

In recombinant DNA technology the ultimate aim is to produce the required product in large amount. The gene for the product cloned into the cells or the organisms must be induced to express in the form of a protein in large amounts. Bio-reactors are used for this. Bio-reactors may be 1. Simple stirrer-tank bioreactor 2. Sparge stirred-tank bioreactor.

Parts of the bio-reactor: 1. A large metallic or glass tank. 2. Stirrer 3. Inlets for medium, steam and sterile air 4. Pressure gauge 5. Out let for product.



- A stirred-tank reactor is usually cylindrical or with a curved base to facilitate the mixing of the reactor contents. It holds the medium
- The stirrer facilitates even mixing and oxygen availability throughout the bioreactor. Alternatively air can be bubbled through the reactor.
- The bioreactor has an agitator system attached to a motor.
- An oxygen delivery system and a foam control system, a temperature control system is attached.
- Inlet for addition of buffer to control pH.
- Sampling ports are used to withdraw small volumes of the culture periodically.
- Pressure gauge controls the pressure inside.

4. What are the different methods of insertion of recombinant DNA into the host cell?

Ans: There are several methods of introducing the ligated DNA into recipient cells.

They are: **1. Transformation method**

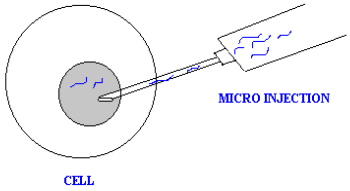
2. Micro-injection method

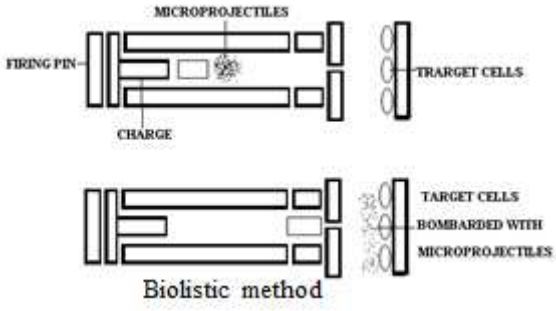
3. Biolistic or Gene gun method

4. 'Disarmed pathogen' Vectors

1. Transformation method: Since DNA is a hydrophilic molecule, it cannot pass through cell membranes. In order to force bacteria to take up the plasmid, the bacterial cells must first be made 'competent' to take up DNA. This is done by treating them with a specific concentration of a divalent cation, such as calcium, which increases the efficiency with which DNA enter the bacterium through pores in its cell wall. Recombinant DNA can then be forced into such cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at 42^0C **(heat shock),** and then putting them back on ice. This enables the bacteria to take up the recombinant DNA.

2. Micro-injection method: In this method recombinant DNA is directly injected with the help of fine needles into the nucleus of an animal cell.





3. Biolistic or Gene gun method: In this method, suitable for plants, cells are bombarded with high velocity micro-particles of gold or tungsten coated with DNA.

4. 'Disarmed pathogen' Vectors: In this method 'disarmed pathogen' vectors, are allowed to infect the cell, transfer the recombinant DNA into the host. ***Agrobacterium tumifacience*** bacterium is used with its **Ti plasmid DNA** as vector in plants.

Long Answer Questions

1. Explain briefly the various processes of recombinant DNA technology?

Ans: Recombinant DNA technology involves several steps in specific sequence such as

1. Isolation of DNA

2. Fragmentation of DNA by restriction endonucleases

3. Isolation of a desired DNA fragment

4. Ligation of the DNA fragment into a vector

5. Transferring the recombinant DNA into the host

6. Culturing the host cells in a medium at large scale and

7. Extraction of the desired product.

1. Isolation of the Genetic Material (DNA):

In order to cut the DNA with restriction enzymes, it needs to be in pure form, free from other macro-molecules. Since the DNA is enclosed within the membranes, we have to break the cell open to release DNA along with other macromolecules such as RNA, proteins, polysaccharides and also lipids. This can be achieved by treating the bacterial cells/plant or

animal tissue with enzymes such as **lysozyme** (bacteria), **cellulase** (plant cells), **chitinase** (fungus).

Genes are located on long molecules of DNA interwined with proteins such as histones. The RNA can be removed by treatment with ribonuclease whereas proteins can be removed by treatment with protease. Other molecules can be removed by appropriate

treatments and purified DNA ultimately precipitates out after the addition of chilled ethanol.

2. Cutting of DNA at Specific Locations (Fragmentation of DNA by restriction endonucleases): Restriction enzyme digestions are performed by incubating purified DNA molecules with the restriction enzyme, at the optimal conditions for that specific enzyme.

Each restriction endonuclease functions by 'inspecting' the length of a DNA sequence. Once it finds its specific recognition sequence, it will bind to the DNA and cut each of the two strands of the double helix at specific points in their sugar-phosphate backbones.

Each restriction endonuclease recognises a specific **palindromic nucleotide sequences** in the DNA.

The palindrome in DNA is a sequence of base pairs that reads same on the two strands when orientation of reading is kept the same. For example, the following sequences reads the same on the two strands in 5′--- 3′ direction.

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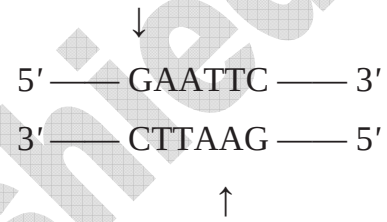

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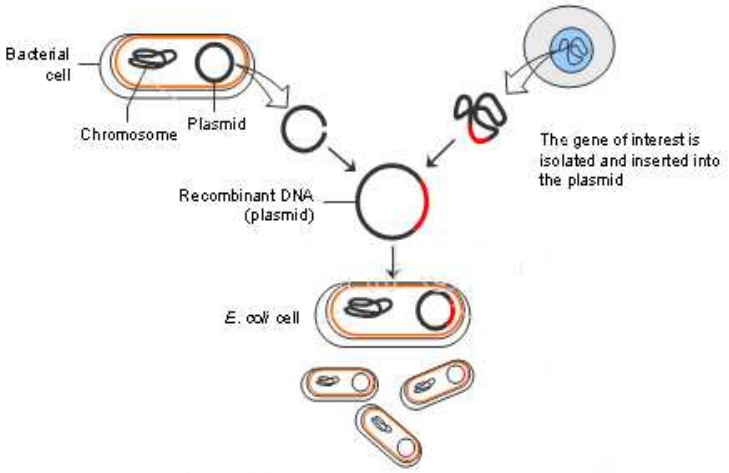

3. Isolation of a desired DNA fragment:

Agarose gel electrophoresis is employed to isolate a desired fragment of DNA. Check the progression of a restriction enzyme digestion.

DNA is a negatively charged molecule, hence it moves towards the positive electrode (anode). The process is repeated with the vector DNA also.

4. Ligation of the DNA fragment into a vector:

The joining of DNA involves several processes. After having cut the source DNA as well as the vector DNA with a specific restriction enzyme, the cut out 'gene of interest' from the source DNA and the cut vector with space are mixed and ligase is added. This results in the preparation of recombinant DNA.

Amplification of Gene of Interest using PCR

PCR stands for **Polymerase Chain Reaction**. In this reaction, multiple copies of the gene (or DNA) of interest is synthesised *in vitro* using two sets of primers and the enzyme DNA polymerase.

The enzyme extends the primers using the nucleotides provided in the reaction and the genomic DNA as template. If the process of replication of DNA is repeated many times, the segment of DNA can be amplified to approximately billion times, i.e., 1 billion copies are made. The amplified fragment if desired can now be used to ligate with a vector for further cloning.

5. Insertion of Recombinant DNA into the Host Cell/Organism (Transferring the recombinant DNA into the host):

There are several methods of introducing the ligated DNA into recipient cells. Recipient cells after making them 'competent' to receive, take up DNA present in its surrounding.

Methods like **1. Transformation method**

2. Micro-injection method

3. Biolistic or Gene gun method

4. 'Disarmed pathogen' Vectors

Can be employed to transfer the DNA into the host.

Screening for Recombinants: Many method are used to screen for the recombinants like antibiotic resistance, colony hybridization, inactivated due to insertion etc.

If a recombinant DNA bearing gene for resistance to an antibiotic (e.g., ampicillin) is transferred into *E. coli* cells, the host cells become transformed into ampicillin-resistant cells. If we spread the transformed cells on agar plates containing ampicillin, only transformants will grow, untransformed recipient cells will die. Since, due to ampicillin resistance gene, one is able to select a transformed cell in the presence of ampicillin. The ampicillin resistance gene in this case is called a **selectable marker**.

6. Obtaining the Foreign Gene Product (Culturing the host cells in a medium at large scale):

When we insert a piece of alien DNA into a cloning vector and transfer it into a bacterial, plant or animal cell, the alien DNA gets multiplied. In almost all recombinant technologies, the ultimate aim is to produce a desirable protein. Hence, there is a need for the recombinant DNA to be expressed. The foreign gene gets expressed under appropriate conditions.

After having cloned the gene of interest and having optimised the conditions to induce the expression of the target protein and producing it on a large scale. If any protein encoding gene is expressed in a heterologous host, it is called a **recombinant protein**.

The cells harbouring cloned genes of interest may be grown on a small scale in the laboratory or **bio-reactors**. To produce in large quantities, the development of **bioreactors**, where large volumes (100-1000 litres) of culture can be processed, was required.

7. Extraction of the desired product:

The cultures may be used for extracting the desired protein and then purifying it by using different separation techniques.

The cells can also be multiplied in a continuous culture system wherein the used medium is drained out from one side while fresh medium is added from the other to maintain the cells in their physiologically active log/exponential phase. This type of culturing method produces a larger biomass leading to higher yields of desired protein.

Small volume cultures cannot yield appreciable quantities of products.

Downstream Processing

After completion of the biosynthetic stage, the product has to be subjected through a series of processes before it is ready for marketing as a finished product.

The processes include separation and purification, which are collectively referred to as downstream processing. The product has to be formulated with suitable preservatives. Such formulation has to undergo thorough clinical trials as in case of drugs. Strict quality control testing for each product is also required. The downstream processing and quality control testing vary from product to product.

2. Give a brief account of the tools of recombinant DNA technology?

Ans: Genetic engineering or recombinant DNA technology can be accomplished only if we have the key tools, i.e., **restriction enzymes, polymerase enzymes, ligases, vectors and the host organism**.

1. Restriction Enzymes

Restriction enzymes belong to a larger class of enzymes called **nucleases**.

These are of two kinds; **exonucleases** and **endonucleases**.

Exonucleases remove nucleotides from the ends of the DNA whereas, endonucleases make cuts at specific positions within the DNA.

Each restriction endonuclease functions by 'inspecting' the length of a DNA sequence. Once it finds its specific recognition sequence, it will bind to the DNA and cut each of the two strands of the double helix at specific points in their sugar-phosphate backbones .

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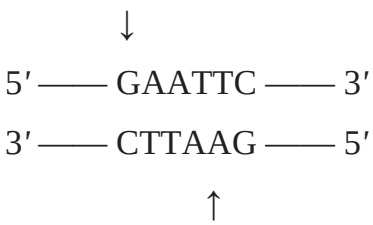

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This stickiness of the ends facilitates the action of the enzyme **DNA ligase**.

Restriction endonucleases are used in genetic engineering to form **'recombinant'** molecules of DNA. Which are composed of DNA from different sources/genomes.

When cut by the same restriction enzyme, the resultant DNA fragments have the same kind of 'sticky-ends' and, these can be joined together using DNA ligases.

2. Cloning Vectors:

Plasmids , bacteriophages and cosmids have the ability to replicate within bacterial cells independent of the control of chromosomal DNA. These are the commonly used vectors.

Bacteriophages because of their high number per cell, have very high copy numbers of their genome within the bacterial cells.

Plasmids are extra chromosomal self replicating circular DNA molecules present in many bacteria.

Some plasmids may have only one or two copies per cell whereas others may have 15-100 copies per cell. Their numbers can go even higher. If we are able to link an alien piece of DNA with bacteriophage or plasmid DNA, one can multiply its numbers equal to the copy number of the plasmid or bacteriophage.

Apart from these artificial synthesized plasmids like **pBR322**, **pUC 19,101** are also popular.

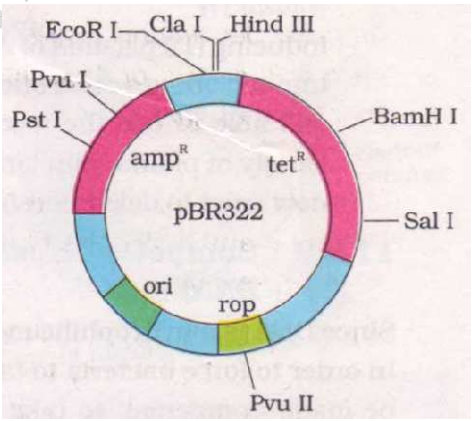
Unless one cuts the vector and the source DNA with the same restriction enzyme, the recombinant vector molecule cannot be created.

Vectors used at present, are engineered in such a way that they help easy linking of foreign DNA and selection of recombinants from non-recombinants.

The following are the features that are required to facilitate cloning into a vector.

a) Origin of Replication (ori):

This is a sequence from where replication starts and any piece of DNA when linked to this sequence can be made to replicate within the host cells. This sequence is also responsible for controlling the copy number of the linked DNA. So, if one wants to recover many copies of the target DNA it should be cloned in a vector whose origin support high copy number.



b) Selectable Marker*:*

In addition to 'ori', the vector requires a selectable marker, which helps in identifying and eliminating nontransformants and selectively permitting the growth of the transformants. **Transformation** is a procedure through which a piece of DNA is introduced in a host bacterium. Normally, the genes encoding resistance to antibiotics such as ampicillin, , tetracycline, chloramphenicol or kanamycin, etc., are considered useful selectable markers for *E. coli.*

The normal *E. coli* cells do not carry resistance against any of these antibiotics.

c) Cloning Sites:

In order to link the alien DNA, the vector needs to have very few, preferably single, **recognition sites** for the commonly used restriction enzymes. Presence of more than one recognition sites within the vector will generate several fragments, which will complicate the gene cloning.

d) Molecular Weight:

The cloning vector should have low molecular weight.

e) Vectors for cloning genes in plants:

Agrobacterium tumifaciens, a pathogen of several dicot plants is able to deliver a piece of DNA known as **' T-DNA'** to transform normalplant cells into a tumor and direct these tumor cells to produce the chemicals required by the pathogen. The tumor inducing **(Ti)** plasmid of *Agrobacterium tumifaciens* has now been modified into a cloning vector which is no more pathogenic to the plants but is still able to use the mechanisms to deliver genes of our interest into a variety of plants.

3. Competent Host (For Transformation with Recombinant DNA)

Since DNA is a hydrophilic molecule, it cannot pass through cell membranes.In order to force bacteria to take up the plasmid, the bacterial cells must first be made 'competent' to take up DNA. This is done by treating them with a specific concentration of a divalent cation,

such as calcium, which increases the efficiency with which DNA enters the bacterium through pores in its cell wall. Recombinant DNA can then be forced into such cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at 420C (heat shock), and then putting them back on ice. This enables the bacteria to take up the recombinant DNA.

Method known as micro-injection, biolistics or gene gun, '**disarmed pathogen'** vectors, which when allowed to infect the cell, transfer the recombinant DNA into the host.
These are the tools for constructing recombinant DNA.